

## **Thromboxane A<sub>2</sub> Receptor Is Highly Expressed in Mouse Immature Thymocytes and Mediates DNA Fragmentation and Apoptosis**

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### **Summary**

We have recently revealed that the thymus is the organ showing the highest expression of thromboxane (TX) A<sub>2</sub> receptor in mice. In this study, thymic cell populations expressing the receptor were identified, and the effects of a TXA<sub>2</sub> agonist on these cells were examined. Radioligand binding using a TXA<sub>2</sub> receptor-specific radioligand revealed a single class of binding sites in the thymocytes with an affinity and specificity identical to those reported for the TXA<sub>2</sub> receptor. The receptor density in these cells was comparable to that seen in blood platelets. This receptor is most highly expressed in CD4<sup>-</sup>8<sup>-</sup> and CD4<sup>+</sup>8<sup>+</sup> immature thymocytes, followed by CD4<sup>+</sup>8<sup>-</sup> and CD4<sup>-</sup>8<sup>+</sup> cells. The receptor density in splenic T cells was less than one fifth of that in CD4<sup>+</sup>8<sup>+</sup> cells and no binding activity was detectable in splenic B cells. The addition of a TXA<sub>2</sub> agonist, STA<sub>2</sub>, to thymocytes induced the disappearance of the CD4<sup>+</sup>8<sup>+</sup> cells in a time- and concentration-dependent manner and caused DNA fragmentation. These changes were blocked by a specific TXA<sub>2</sub> antagonist, S-145. These results demonstrate that TXA<sub>2</sub> induces apoptotic cell death in immature thymocytes by acting on the TXA<sub>2</sub> receptor on their cell surface and suggest a role for the TXA<sub>2</sub>/TXA<sub>2</sub> receptor system in the thymic micro-environment.

**P**rostanoids such as prostaglandins (PGs), thromboxane (TX), and leukotrienes (LTs) are a family of oxygenated arachidonate metabolites that exert a variety of actions to maintain local homeostasis in the body (1, 2). Their actions as inflammatory mediators are well known; for example, PGE<sub>2</sub> causes vasodilation to increase local blood flow, LTB<sub>4</sub> induces chemotaxis and activates neutrophils, and LTC<sub>4</sub> increases vascular permeability. Some of these compounds are produced upon immunologic challenge and are mediators of anaphylaxis. A recent report also suggests the participation of PGE<sub>2</sub> to the apoptotic cell death of ovarian surface epithelial cells (3). However, little is known about their action in the thymus. TXA<sub>2</sub> is a very unstable arachidonate metabolite with a half life of about 30 s under physiological conditions. Yet, it is a potent stimulator of platelets and a constrictor of vascular and respiratory smooth muscles, and is presumed to play a role in thrombosis, myocardial infarction, and bronchial asthma (4). This compound is synthesized by a specific enzyme, TX synthase, and functions through a cell surface receptor. We have cloned cDNAs for the human and mouse TXA<sub>2</sub> receptors and revealed that they belong to the family of rhodopsin-

type receptors (5, 6). Northern blot analysis in various mouse organs showed that the TXA<sub>2</sub> receptor mRNA is most abundantly expressed in the thymus, followed by the spleen and lung (6). On the other hand, TX synthase is richest in platelets, followed by macrophages (7). In the thymus, it is present in the cortical epithelial cells and dendritic cells of the medulla (8, 9). These findings urged us to investigate the localization of the TXA<sub>2</sub> receptor and its possible role in the thymus. In this study, using a radioligand binding assay, we determined the distribution of the TXA<sub>2</sub> receptor in cells of the murine thymus and spleen, and found that this receptor is predominantly expressed in immature CD4<sup>-</sup>8<sup>-</sup> and CD4<sup>+</sup>8<sup>+</sup> thymocytes. We further found that a TXA<sub>2</sub> agonist evokes DNA fragmentation and apoptosis in these cells.

### **Materials and Methods**

*Materials.* 5Z-7-(3-endo-phenylsulphonylamino-bicyclo[2.2.1]hept-2-exo-yl) heptenoic acid (S-145) and [<sup>3</sup>H]S-145 were gifts from Shionogi Research Laboratories (Osaka, Japan). 9,11-epithio-11,12-methano-thromboxane A<sub>2</sub> (STA<sub>2</sub>), 9,11-dimethylmethano-11,12-

methano-13,14-dihydro-13-aza-14-oxo-15-cyclopentyl-16,17,18,19, 20-pentano-15-epi-thromboxane A<sub>2</sub> (ONO3708), PGD<sub>2</sub>, PGF<sub>2α</sub>, PGE<sub>2</sub>, and TXB<sub>2</sub> were gifts from Ono Pharmaceuticals (Osaka, Japan). 15(S)-hydroxy-11,9-epoxymethano-prosta-5Z,13E-dienoic acid (U46619) and iloprost were obtained from the Upjohn Company (Kalamazoo, MI) and Amersham International (Amersham, England), respectively. Phycoerythrin-conjugated anti-CD4 and fluorescein isothiocyanate-conjugated anti-CD8 mAbs were purchased from Becton Dickinson & Co. (Mountain View, CA). Diphenylamine was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of reagent grade.

**Cell Preparation and Culture.** BALB/c mice of 5–7 wk-of-age were purchased from Japan SLC, Inc. (Shizuoka, Japan) and used after at least 1 wk of maintenance in our laboratory. Thymocyte suspensions were prepared in Eagle's MEM by mincing the thymus and filtering dissociated cells through a nylon mesh (pore size, 40 μm) (10). To examine the distribution of the TXA<sub>2</sub> receptor, the cells were initially treated with either anti-CD4 or anti-CD8 Ab or both followed by treatment with rabbit complement, and separated into fractions of CD4<sup>+</sup>8<sup>+</sup> plus CD4<sup>+</sup>8<sup>-</sup> cells, CD4<sup>+</sup>8<sup>-</sup> cells plus CD4<sup>-</sup>8<sup>-</sup> cells, and CD4<sup>-</sup>8<sup>-</sup> cells (11). Splenic T cells were obtained by passing dissociated cells successively through a nylon wool column and a Sephadex G-10 column. Splenic B cells were obtained as reported previously (11). The purity of the splenic cell fractions was in the range of 95–99%. In some experiments, CD4<sup>+</sup>8<sup>+</sup> cells were sorted using a cell sorter (ABCAS-100; Showa Denko Co. Ltd., Tokyo, Japan).

**Binding Assay.** Cells, washed once with phosphate buffer (10 mM sodium phosphate, pH 7.4, containing 10 mM EDTA, 5 mM KCl, and 135 mM NaCl), were suspended in Hepes-saline (20 mM Hepes, 5 mM KCl, 5 mM MgCl<sub>2</sub>, and 140 mM NaCl, pH 7.4) and the binding assay was carried out as reported previously (12). Cells were incubated with various concentrations (0.5–8 nM) of [<sup>3</sup>H]S-145 for Scatchard analysis, or with 5 nM [<sup>3</sup>H]S-145 and various concentrations of TXA<sub>2</sub> analogs and PGs in the displacement experiments. Nonspecific binding was determined as the binding in the presence of 20 μM unlabeled S-145.

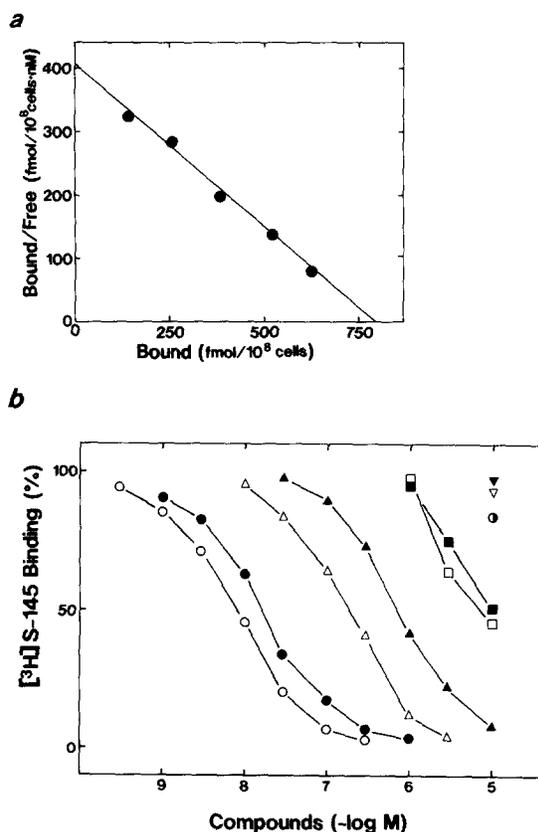
**DNA Fragmentation.** Thymocytes were washed once with Eagle's MEM and suspended at 1.8–5.3 × 10<sup>7</sup> cells/ml in RPMI 1640 containing 1% BSA. The cells were then incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in the presence of relevant agents for 20 h. DNA fragmentation was measured as described by Wyllie (13). Briefly, the cells were lysed with 0.5% Triton X-100, and centrifuged at 27,000 g to separate fragmented DNA in the supernatant from chromatin in the pellet. DNA in the supernatant and pellet was then precipitated by the addition of trichloroacetic acid and quantified using the diphenylamine reaction (14). The percent fragmentation indicates the ratio of DNA in the supernatant to the total DNA recovered in the supernatant and pellet. The values are presented after subtracting DNA fragmentation in the control experiment, which was 10–15% on average.

**Flow Cytometry.** Thymocytes were incubated at 37°C in RPMI 1640 containing 10% FCS with or without relevant agents for the indicated periods. The cells were stained with phycoerythrin-conjugated anti-CD4 and fluorescein isothiocyanate-conjugated anti-CD8 mAbs. Approximately 5 × 10<sup>4</sup> cells were analyzed by a FACScan<sup>®</sup> two-color flow cytometer (Becton Dickinson & Co.) as reported previously (10, 11).

## Results and Discussion

**The TXA<sub>2</sub> Receptor Is Highly Expressed in Immature Thymocytes.** A thymocyte suspension was prepared from 5–7-

wk-old BALB/c mice and used in the binding assay for the TXA<sub>2</sub> receptor employing [<sup>3</sup>H]S-145 as a radioligand (12, 15). Scatchard analysis of the results showed a single class of binding sites with K<sub>d</sub> and B<sub>max</sub> values of 2.03 ± 0.15 nM and 788 ± 15 fmol/10<sup>8</sup> cells (~4,800 sites/cell), respectively (Mean ± SEM, n = 4) (Fig. 1 a). This [<sup>3</sup>H]S-145 binding was effectively displaced by TXA<sub>2</sub> antagonists and agonists with a rank order of potency of S-145 > ONO3708 > STA<sub>2</sub> > U46619. The binding was partially displaced by a PGI<sub>2</sub> agonist, iloprost, and PGD<sub>2</sub>, while TXB<sub>2</sub>, PGE<sub>2</sub>, and PGF<sub>2α</sub> were ineffective at concentrations up to 10 μM (Fig. 1 b). The affinity and specificity of binding were identical to respective values reported for the TXA<sub>2</sub> receptor in other cells (12, 15–17). These results indicated that this binding activity represents the TXA<sub>2</sub> receptor, and that the thymocytes express this receptor at a density comparable to that found in blood platelets, ~2,000 sites/cell (18, 19). By incubation in buffer alone or with anti-CD4 Ab, anti-CD8 Ab, or both Abs, the thymocytes were fractionated into four fractions according to their expression of CD4 and CD8 (11). A cell suspension was also prepared from mouse spleen cells and



**Figure 1.** Binding of a TXA<sub>2</sub> receptor-specific radioligand, [<sup>3</sup>H]S-145, to mouse thymocytes. (a) Scatchard analysis. (b) Specificity of [<sup>3</sup>H]S-145 binding to thymocytes. A whole thymocyte suspension was prepared and ligand binding was carried out as described in Materials and Methods. Displacement curves are shown for various compounds; S-145 (○), ONO3708 (●), STA<sub>2</sub> (△), U46619 (▲), Iloprost (□), PGD<sub>2</sub> (■), PGF<sub>2α</sub> (⊙), PGE<sub>2</sub> (▽), and TXB<sub>2</sub> (▼). Mean values (n = 3) are shown.

separated into T and B cell fractions as described previously (11). These cell fractions were then used in the binding assay and the density of the TXA<sub>2</sub> receptor in each lymphocyte population was calculated. As shown in Table 1, CD4<sup>-</sup>8<sup>-</sup> and CD4<sup>+</sup>8<sup>+</sup> thymocytes expressed the highest level of receptor, which was about twice that of CD4<sup>+</sup>8<sup>-</sup> and CD4<sup>-</sup>8<sup>+</sup> cells and more than five times that of splenic T cells. The TXA<sub>2</sub> receptor was not detected in B cells with the method used. These results clearly show that the TXA<sub>2</sub> receptor is expressed early in development of T cells in the thymus and its expression decreases gradually during maturation. It is not clear from these results, however, whether the receptor is expressed in all CD4<sup>-</sup>8<sup>-</sup> and CD4<sup>+</sup>8<sup>+</sup> cells or localized to some subpopulation(s) of these cells.

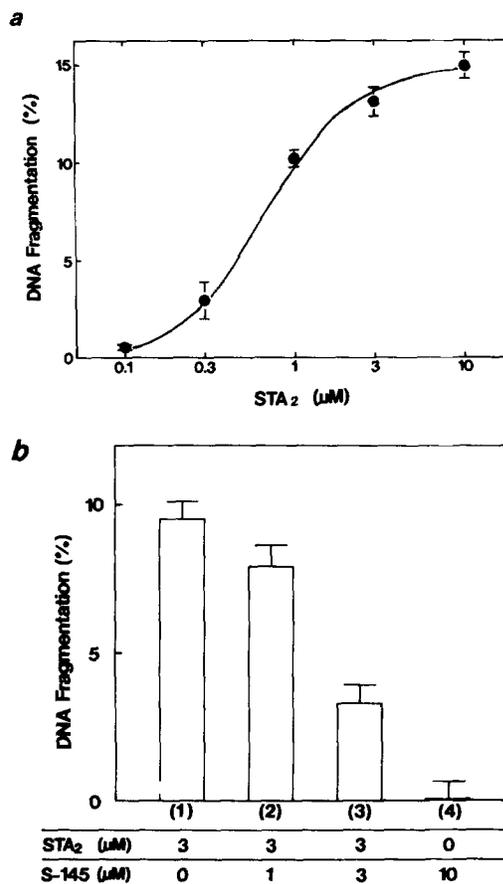
*A TXA<sub>2</sub> Agonist, STA<sub>2</sub>, Induces DNA Fragmentation in Thymocytes and a TXA<sub>2</sub> Antagonist, S-145, Inhibits this Action.* The preceding results suggest that TXA<sub>2</sub> can act on thymocytes and induce some biological response. TXA<sub>2</sub> is known to evoke phosphatidyl inositol breakdown through a GTP binding protein and to induce a rise in the intracellular Ca<sup>2+</sup> concentration and activate protein kinase C (20, 21). Because several reports (13, 22–24) implicated a rise in Ca<sup>2+</sup> concentration and/or protein kinase C activation in DNA fragmentation and apoptosis of immature thymocytes, we added a TXA<sub>2</sub> agonist, STA<sub>2</sub> (18, 25), to thymocytes and examined its effects. As shown in Fig. 2 a, STA<sub>2</sub> induced DNA fragmentation of thymocytes in a concentration-dependent manner in 20 h of incubation. This effect was half maximal at ~0.7 μM STA<sub>2</sub> and reached a plateau at 10 μM, the concentration at which 15% of the total DNA was frag-

**Table 1.** Binding of [<sup>3</sup>H]S-145 to Lymphocyte Populations of Mouse Thymus and Spleen

Organ	Population	[ <sup>3</sup> H]S-145 Binding
		<i>fmol/10<sup>8</sup> cells<sup>†</sup></i>
Thymus*	Whole T cells	788 ± 15
	CD4 <sup>-</sup> 8 <sup>-</sup> cells	934 ± 167
	CD4 <sup>+</sup> 8 <sup>+</sup> cells	1178 ± 74
	CD4 <sup>+</sup> 8 <sup>-</sup> cells	481 ± 52
	CD4 <sup>-</sup> 8 <sup>+</sup> cells	404 ± 38
Spleen	T cells	174 ± 15
	B cells	<60

\* Lymphocytes were prepared from mouse thymus and separated into fractions of CD4<sup>-</sup>8<sup>+</sup> plus CD4<sup>-</sup>8<sup>-</sup> cells, CD4<sup>+</sup>8<sup>-</sup> plus CD4<sup>-</sup>8<sup>-</sup> cells, and CD4<sup>-</sup>8<sup>-</sup> cells as described in Materials and Methods. A [<sup>3</sup>H]S-145 binding study was carried out in these fractions and unfractionated cell suspensions. Proportions of the CD4<sup>-</sup>8<sup>-</sup>, CD4<sup>+</sup>8<sup>+</sup>, CD4<sup>+</sup>8<sup>-</sup>, and CD4<sup>-</sup>8<sup>+</sup> cells in these fractions were determined by flow cytometric analysis using anti-CD4 and anti-CD8 mAbs. The binding activity in each cell population was then calculated from binding values in the above fractions on the basis of their proportions.

† Mean ± SEM are shown (*n* = 3–4).



**Figure 2.** Induction of DNA fragmentation by a specific TXA<sub>2</sub> agonist, STA<sub>2</sub>. (a) A concentration-effect curve. Mean ± SEM are shown (*n* = 4). (b) Inhibition by a specific TXA<sub>2</sub> antagonist, S-145. Mean ± SEM are shown (*n* = 3).

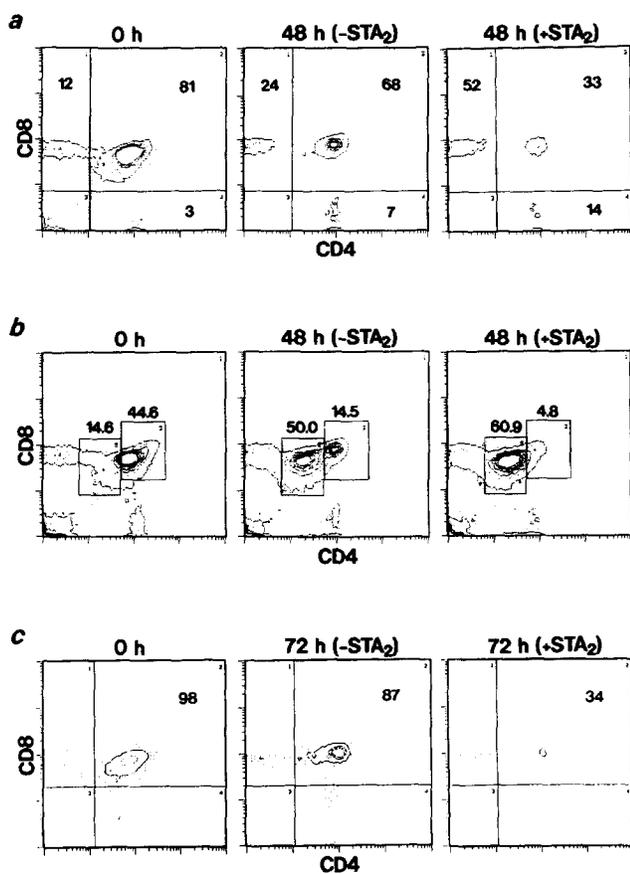
mented. Under this condition, the cell viability, examined by the trypan blue dye exclusion method, decreased to 81.6 ± 1.3% compared with 98.5 ± 1.1% in the control (mean ± SEM, *n* = 13). This dose-dependency correlated well with that observed for platelet aggregation to STA<sub>2</sub> in platelet rich plasma (18). The STA<sub>2</sub>-induced DNA fragmentation was inhibited in a concentration-dependent manner by a specific TXA<sub>2</sub> antagonist, S-145 (26) (Fig. 2 b), suggesting that it was a receptor-mediated process. Actinomycin D, 2 μg/ml, completely inhibited the STA<sub>2</sub>-induced increase in DNA fragmentation, suggesting the involvement of protein synthesis in this process as reported for steroid-induced DNA fragmentation (27). An inactive metabolite of TXA<sub>2</sub>, TXB<sub>2</sub>, did not induce DNA fragmentation. Some other prostaglandins, PGD<sub>2</sub> and PGF<sub>2α</sub> at 3 μM concentration had no effect, whereas PGE<sub>2</sub> induced DNA fragmentation comparable to that seen with STA<sub>2</sub>; 16% fragmentation at 3 μM. Because the affinity of PGE<sub>2</sub> for the TXA<sub>2</sub> receptor was very low (Fig. 1 b), it probably acted on other types of PG receptors. We have recently cloned cDNAs for the EP<sub>2</sub> and EP<sub>3</sub> subtypes of the PGE<sub>2</sub> receptor, and found significant expression of their mRNAs in the mouse thymus (28, 29).

Contrary to the effect on thymocytes, STA<sub>2</sub> could not induce DNA fragmentation in splenic T cells (data not shown).

**STA<sub>2</sub> Causes Apoptosis in CD4<sup>+</sup>8<sup>+</sup> Thymocytes.** The above results clearly showed that a TXA<sub>2</sub> agonist acted on a specific receptor and induced DNA fragmentation and cell death in thymocytes. To identify the thymocyte subpopulations affected by this treatment, we carried out flow cytometric analysis using anti-CD4 and anti-CD8 antibodies. As shown in Fig. 3 *a*, this analysis revealed that the proportion of viable CD4<sup>+</sup>8<sup>+</sup> cells was preferentially decreased by STA<sub>2</sub> treatment, while the proportions of CD4<sup>+</sup>8<sup>-</sup> and CD4<sup>-</sup>8<sup>+</sup> cells increased. S-145 significantly inhibited the STA<sub>2</sub>-induced decrease in the CD4<sup>+</sup>8<sup>+</sup> cell population (data not shown). The effects of the STA<sub>2</sub> on the CD4<sup>-</sup>8<sup>-</sup> cells could not be determined due to their small numbers. A similar analysis was then performed on a cell population containing dead

cells, since Swat et al. (30) recently reported that the expression of CD4 and CD8 molecules on the CD4<sup>+</sup>8<sup>+</sup> cells was reduced during apoptosis and that this change could be detected when dead cells were included in the analysis. Therefore, we examined whether a similar change occurred during STA<sub>2</sub> treatment. As shown in Fig. 3 *b*, upon culturing thymocytes in suspension, a distinct subpopulation with decreased expression of CD4 and CD8 molecules appeared in a time-dependent manner in the CD4<sup>+</sup>8<sup>+</sup> population, and this subpopulation was increased significantly by the addition of STA<sub>2</sub>. These results indicated that STA<sub>2</sub> induced apoptosis in CD4<sup>+</sup>8<sup>+</sup> cells. To confirm this, we isolated the CD4<sup>+</sup>8<sup>+</sup> cells by cell sorting and tested the effect of STA<sub>2</sub>. As shown in Fig. 3 *c*, when viable cells were analyzed, the addition of 10 μM STA<sub>2</sub> accelerated the disappearance of double-positive cells. These results strongly suggest that only immature CD4<sup>+</sup>8<sup>+</sup> thymocytes are sensitive to TXA<sub>2</sub>, and they undergo apoptosis on exposure to the agonist. On the other hand, CD4<sup>+</sup>8<sup>-</sup> and CD4<sup>-</sup>8<sup>+</sup> cells did not undergo apoptosis, although they express significant amounts of the TXA<sub>2</sub> receptor. A similar difference in sensitivity between immature and mature thymocytes has been reported in response to anti-CD3 Ab stimulation (23) and this may be due to the presence of a system preventing apoptosis such as *bcl-2* gene product (31, 32) in the mature cells.

The thymic microenvironment is thought to be very important for T cell development. It is presumed to provide cell to cell interactions and soluble factors to pursue maturation and selection of T cells. However, neither cell interactions nor soluble factors responsible for these processes have been fully characterized or identified. In this study we found that the TXA<sub>2</sub> receptor is highly expressed in immature thymocytes and mediates the apoptosis of these cells. As demonstrated by immunohistochemistry, TX synthase is present in reticular epithelial cells and dendritic cells but not in lymphocytes of the thymus (8, 9). In fact, isolated thymic interdigitating cells actually produce TXA<sub>2</sub> (33), while lymphocytes including thymocytes show negligible cyclooxygenase activity (34, 35). These results strongly suggest that TXA<sub>2</sub> works as a stromal cell-derived endogenous paracrine signal in the thymus and is involved in thymocyte maturation and selection. Because prostanoids including TXA<sub>2</sub> are synthesized and released only upon cell stimulation and TXA<sub>2</sub> is a very unstable compound with a half life of 30 s under physiological conditions, we expect that it acts on lymphocytes only in close vicinity to or in direct contact with cells stimulated to produce TXA<sub>2</sub>. Such a property of the TXA<sub>2</sub>/TXA<sub>2</sub> receptor system fits very well with the proposed character of the thymic microenvironment. It is already known that other signals such as those acting on the CD3-T cell receptor complex (23), the Fas antigen (36, 37), and integrins (38) are also involved in T cell maturation and selection. Thus, different mechanisms appear to work at different stages of these processes under different conditions. The TXA<sub>2</sub>/TXA<sub>2</sub> receptor system may play a role in one or some of these stages, and work independently or in combination with other signals. Many growth signals serve as both positive and negative regulators of growth, dependent on the



**Figure 3.** Flow cytometric analyses of mouse thymocyte populations after treatment with a specific TXA<sub>2</sub> agonist, STA<sub>2</sub>. (a) and (b) Whole thymocytes were incubated for 48 h, stained with fluorescence-labeled anti-CD4 and anti-CD8 antibodies, and analyzed by a FACScan® two-color flow cytometer as described in Materials and Methods. The numbers in the figures represent the percentage of cells included in each quadrant. 0 h, cells before incubation. 48 h (-STA<sub>2</sub>) and 48 h (+STA<sub>2</sub>), cells treated without or with STA<sub>2</sub> for 48 h, respectively. Only viable cells (a) or both viable and dead cells (b) were gated by forward and side-scatter. (c) Analysis on an isolated CD4<sup>+</sup>8<sup>+</sup> cell population. CD4<sup>+</sup>8<sup>+</sup> cells were sorted with a cell sorter and incubated with or without 10 μM STA<sub>2</sub> for 72 h, and stained as described. Only viable cells were gated for analysis.

situations. Though a TXA<sub>2</sub> agonist induced apoptosis in isolated thymocyte suspension, TXA<sub>2</sub> may behave as a growth signal rather than as an apoptotic signal in the physiological context. Growth stimulatory activity of TXA<sub>2</sub> has already been reported in aortic smooth muscle cells (17) and suggested for peripheral T cells (39). It remains to be determined in what physiological context and at what developmental stage the TXA<sub>2</sub>/TXA<sub>2</sub> receptor system acts in the thymus. TXA<sub>2</sub> is synthesized through sequential reactions

by cyclooxygenase and TX synthase, and it has been shown that TIS10, a cyclooxygenase that can be induced by cytokines and growth factors, is highly expressed in the thymus of the neonatal mouse and disappears quickly after birth (40, 41). The precise role of the TXA<sub>2</sub>/TXA<sub>2</sub> receptor system will be clarified by determining the physiological stimuli required to evoke TXA<sub>2</sub> production in the thymus, and the present report should facilitate studies on its role for intrathymic T cell development.

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